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Genetic diversity in introduced Douglas-fir and its natural regeneration in Central Europe

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Since its first introduction in the 19th century, Douglas-fir has become the economically most important non-native forest tree species in Central European countries. Many of these planted forests are important seed sources and/or exhibit natural regeneration. Thus, it is important to assess (1) the genetic diversity of the mature stands and (2) if the genetic diversity can be passed on to the next generations. In order to address these issues, we genotyped mature Douglas-fir individuals and natural regeneration from >100 native and non-native populations using nuclear microsatellite markers. We compared the genetic diversity of native North American populations with mature Douglas-fir populations in Central Europe. The results show that genetic diversity did not differ significantly between European populations and the assigned native origin. Using a subset of 36 sites from Central Europe, we detected a significant reduction in the genetic diversity of adult versus naturally regenerated juvenile trees, indicating a bottleneck effect in the next generation of European Douglas-fir stands. The main reason may be that the mature European Douglas-fir stands are highly fragmented and thus the stand size is not adequate for transmitting the genetic diversity to the next generation. This should be taken into account for the commercial harvesting of seed stands. Seed orchards may offer a potential alternative in providing high quality and genetically diverse reproductive material.

Introduction

In many central European countries, Douglas-fir (*Pseudotsuga menziesii* Mirb. Franco) is the most important introduced tree species in terms of forest area and timber production (Bastien *et al*., 2013; van Loo and Dobrowolska, 2019). Its introduction to Europe dates back to 1827 (Lavender and Hermann, 2014). During the second half of the 19th century, the interest of foresters across the continent increased continuously due to its excellent growth (Locke, 1987; Kownatzki *et al*., 2011; Lavender and Hermann, 2014). Since then, extensive forest areas in Europe have been planted with Douglas-fir, and a large amount of seeds have been used for these afforestations (De Champs, 1997; Kownatzki *et al*., 2011). After more than a century of silvicultural history, these stands are the main seed source for most European countries (Konnert *et al*., 2018). However, for many stands, the native origin has not been documented. Furthermore, the genetic diversity of these stands and its potential transfer to the next generation of European Douglas-fir stands remain largely unexplored (Hintsteiner *et al*., 2018; Wojacki *et al*., 2019).

Within the area of natural distribution in Western North America, Douglas-fir covers a wide latitudinal (18°–55°) and altitudinal (0–3260 m above sea level) range (Lavender and Hermann, 2014). The coastal (*P. menziesii* var. *menziesii*) and interior or Rocky Mountain (*P. menziesii* var. *glauca*) varieties display significant differentiation of quantitative (Eilmann *et al*., 2013; Chakraborty *et al*., 2016) and physiological traits (Zavarin and Snajberk, 1973), as well as at putatively selectively neutral (Krutovsky *et al*., 2009; Neophytou *et al*., 2016) and candidate gene loci (Müller *et al*., 2015). Genetic differentiation between, but also within the two varieties has been shaped both by topography and demographic history (Gugger *et al*., 2010; Gugger and Sugita, 2010; Wei *et al*., 2011; van Loo *et al*., 2015). High levels of genetic diversity can be found in areas of former glacial refugia, which hosted large populations and reside at mid-latitudes (Li and Adams, 1989; Klumpp, 1999; Krutovsky *et al*., 2009, Neophytou *et al*., 2016). In contrast, small and isolated populations, mainly towards the edge of the native range, have often undergone population bottlenecks which led to genetic drift and loss of genetic diversity (Li and Adams, 1989). Given this highly differentiated

gene pool, seed origin is expected to be one of the main factors shaping the genetic diversity of introduced Douglas-fir populations. Assignment methods have recently allowed origin identification of many European Douglas-fir stands (Hintsteiner *et al*., 2018).

Besides the native origin, the process of introduction but also subsequent evolution in the introduced range are important factors that shape the gene pool of introduced forest tree populations (Lefèvre, 2004). During the introduction, a limited size of founding population (e.g. use of seeds from a limited number of native trees) may cause genetic depauperation in the introduced populations (Dlugosch and Parker, 2008). Thus far, comparisons based on a limited number of introduced and native populations do not support such founder effects in the first generation of Douglas-fir in Europe (Hoffmann, 1994; Klumpp, 1999). On the other hand, introduction in a new environment lacking conspecific individuals may also cause significant genetic changes in later generations (Lefèvre, 2004; Petit *et al*., 2004). In particular, limited mate availability due to a spatial isolation from other populations, but also due to changes in flowering phenology may result in population bottlenecks and inbreeding (Zheng and Ennos, 1999; Lefèvre *et al*., 2004; Aravanopoulos, 2018).

For the production of genetically diverse forest reproductive material, both the genetic diversity among mature trees and a high number of reproducing trees are important (White *et al*., 2007). Therefore, existing laws and regulations set minimum requirements regarding the size and degree of isolation of a seed stand, as well as the minimum number of trees from which seed is harvested (e.g. BLE, 2016; Anonymous, 2018). However, recent studies suggest that both a reduced genetic diversity and effects of inbreeding depression may occur in progenies of European Douglas-fir seed stands more often than in progenies of native seed sources (Eckhart *et al*., 2017; Wojacki *et al*., 2019). If certified seed stands, which fulfil legal requirements, are prone to genetic erosion and inbreeding, then the same issues must be taken into account when Douglas-fir stands in introduced populations are naturally regenerated. This is particularly important since natural regeneration is a commonly used practice in European Douglas-fir forests (Schmid *et al*., 2014).

Even if previous research has dealt with both the comparison of genetic diversity between native and introduced stands of Douglas-fir (Hoffmann, 1994; Klumpp, 1999) as well as with genetic diversity in seed lots or natural regeneration (Fussi *et al*., 2013; Eckhart *et al*., 2017; Wojacki *et al*., 2019), there is a lack of studies on these topics based on a broad basis of populations. For this study, we use a large sample of Douglas-fir populations from the native range in North America and from Central Europe to address the following tasks: (1) to quantify the genetic diversity across native and introduced Douglas-fir populations in Europe, (2) to compare the genetic diversity of introduced stands with their native origin and (3) to address whether adult trees and natural regeneration from European sites differ in their genetic diversity.

Materials and methods

Study populations

In total, we used genotypic data from (1) 38 populations from the native range and (2) 67 introduced adult populations of Douglas-fir in Central Europe, as well as (3) 36 juvenile populations resulting from natural regeneration within a subset of the investigated Central European adult populations. The native populations are the same as in van Loo *et al*. (2015) and Hintsteiner *et al*. (2018). They consist of progenies from natural stands in the native range. Bulked seeds from at least 15 mother trees within each population in the native range were used to grow these progenies. Among them, 21 had already been planted in the field in provenance trials; while for the remaining 17 populations, seedlings were grown in the nursery. The set of 67 Douglas-fir stands in Central Europe included 36 stands from Germany and Austria, within which natural regeneration was present. Adult trees and the natural regeneration were sampled using a random sampling design. Trunk cambium was collected from adult trees, while needles were sampled from the regeneration or progenies from native populations. In Table 1, we present the coordinates, the number of sampled adult trees and the number of juvenile populations for each Central European population, as well as the origin of the adult populations (according to Hintsteiner *et al*. 2018). The geographic location of both native and introduced populations is depicted in Figure 1.

Laboratory procedures

The collected tissue was dried in bags with silica gel prior to DNA extraction. DNA was extracted from 15–20 mg (dry weight) of tissue using a commercial extraction kit (OMEGA E.Z.N.A Plant DNA, Doraville, USA) following the manufacturer's instructions. A total of 13 nuclear microsatellites (simple sequence repeats [SSRs]), developed by Slavov *et al*. (2004) were amplified by means of multiplex polymerase chain reactions (PCR) (loci PmOSU_2G12, PmOSU_4A7, PmOSU_3B2, PmOSU_5A8, PmOSU_2D4, PmOSU_1F9, PmOSU_3F1, PmOSU_2D6, PmOSU_1C3, PmOSU_2C2, PmOSU_3B9, PmOSU_3D5 and PmOSU_2D9). We arranged the SSR loci into four combinations and performed multiplex PCRs using the Qiagen Type-it Microsatellite kit for each combination (Qiagen, Hilden, Germany). For further details on PCR multiplexing, we refer to van Loo *et al*. (2015). The PCR program included following steps: initial denaturation at 95°C for 5 min, 28 cycles with denaturation at 95°C for 30 s, primer annealing at an annealing temperature—which was specific for each primer combination (details in van Loo *et al*. 2015)—for 1 min and 30 s, elongation at 72°C for 30 s and a final step at 60°C for 30 min. We performed capillary electrophoresis on an ABI PRISM™ 3100 DNA Genetic Analyzer (Applied Biosystems). To score the amplified fragments, we first used the Genescan 3.7 and Genotyper 2.0 software (Applied Biosystems) for inspecting the electropherograms and to choose the peaks corresponding to alleles. We exported the raw sizes of the peaks (not rounded), which we binned using the TANDEM software (Matschiner and Salzburger, 2009). The resulting genotype tables were used for subsequent data analysis.

Data analysis

For population genetic analysis, we treated the adult and juvenile trees by the site as separate populations. Thus, our data were subdivided into: (1) 38 populations from the native range; (2) 67 introduced adult populations from Central Europe including the

Table 1 Overview of the genotyped populations in Central Europe. Coordinates are given in decimal degrees. The origin (assignment to clusters of native populations) is defined according to Hintsteiner *et al.* (2018). The geographic area covered by each cluster in the native range is depicted in Figure 1. N_{adult} = number of genotyped adult trees used for the population genetic analysis in this paper, $N_{regeneration}$ = number of genotyped individuals from the natural regeneration used for the population genetic analysis in this paper.

Population ID	Latitude	Longitude	Origin	N_{adult}	$N_{regeneration}$
AS01	50.48	6.37	I	21	24
AS02	50.52	6.46	I	26	–
AZ02	49.04	12.56	I	19	20
AZ04	48.64	13.01	I	20	–
AZ06	48.41	13.46	VIII/IX	18	–
AZ07	48.24	13.45	I	19	–
BL01	48.33	15.77	I	20	–
BL02	48.32	15.77	I	22	–
CF01	51.03	8.48	I	20	25
CF02	50.94	8.39	I	15	–
CF03	50.45	9.39	I	26	15
CF04	50.65	9.47	I	26	25
FB01	47.60	16.34	II	26	26
FF01	47.88	8.81	I	26	25
FF02	48.33	8.35	I	16	25
FF03	48.33	8.35	I	16	25
FWW01	47.82	9.72	I	26	–
FWW02	47.77	9.72	I	25	23
GW01	48.35	15.60	I	18	20
H01	48.70	15.58	I	23	–
H02	48.70	15.61	I	20	–
H03	48.62	15.53	I	21	–
H04	48.78	15.57	VIII/IX	22	-
OSF01	49.01	10.46	I	26	19
OSF02	48.99	10.55	I	26	22
OSF03	49.03	10.58	I	26	17
S01	48.45	16.45	I	20	25
S02	47.60	16.34	I	20	21
S03	47.56	16.32	I	20	–
S04	48.51	15.72	I	18	16
S05	48.51	15.73	XI	20	–
S06	48.51	15.72	I	20	–
S07	48.16	14.98	I	20	–
S08	48.18	15.03	I	20	25
S09	47.90	14.82	I	20	25
S10	47.02	15.59	I	20	–
S11	48.75	15.00	I	20	24
S12	48.34	14.72	I	20	25
S13	48.32	14.77	I	20	25
S14	48.17	13.60	I	20	7
S15	47.93	13.41	I	20	24
S16	48.33	14.78	I	20	23
S17	48.52	15.76	I	18	15
S18	48.51	15.72	II	20	19
S19	47.64	16.44	I	20	25
TT01	48.00	9.47	I	26	16
TT02	48.03	9.42	I	26	17
TT03	48.07	9.50	I	20	–
TT04	48.09	9.58	I	19	23
TT05	48.14	9.57	I	26	25
TT06	48.22	9.75	I	26	–

Continued

Table 1 Continued

Population ID	Latitude	Longitude	Origin	N_{adult}	$N_{regeneration}$
UF01	48.05	9.26	I	24	22
UF02	48.22	9.18	I	10	–
UF03	48.75	8.32	I	26	24
UF04	48.04	7.76	I	26	–
UFX1	48.40	8.64	I	24	–
UFX2	48.40	8.64	I	25	–
UFX3	48.21	9.22	I	26	–
UFX4	48.16	9.10	I	25	–
UFX5	48.22	9.09	I	26	–
WBR01	51.08	8.27	I	25	23
WBR02	51.08	8.26	I	25	24
WBR03	51.09	8.46	I	26	–
WBR04	51.11	8.49	I	26	–
Z01	48.28	12.67	I	19	–
Z02	48.28	12.67	I	19	–
Z03	48.32	12.68	I	20	–
Total				1460	784

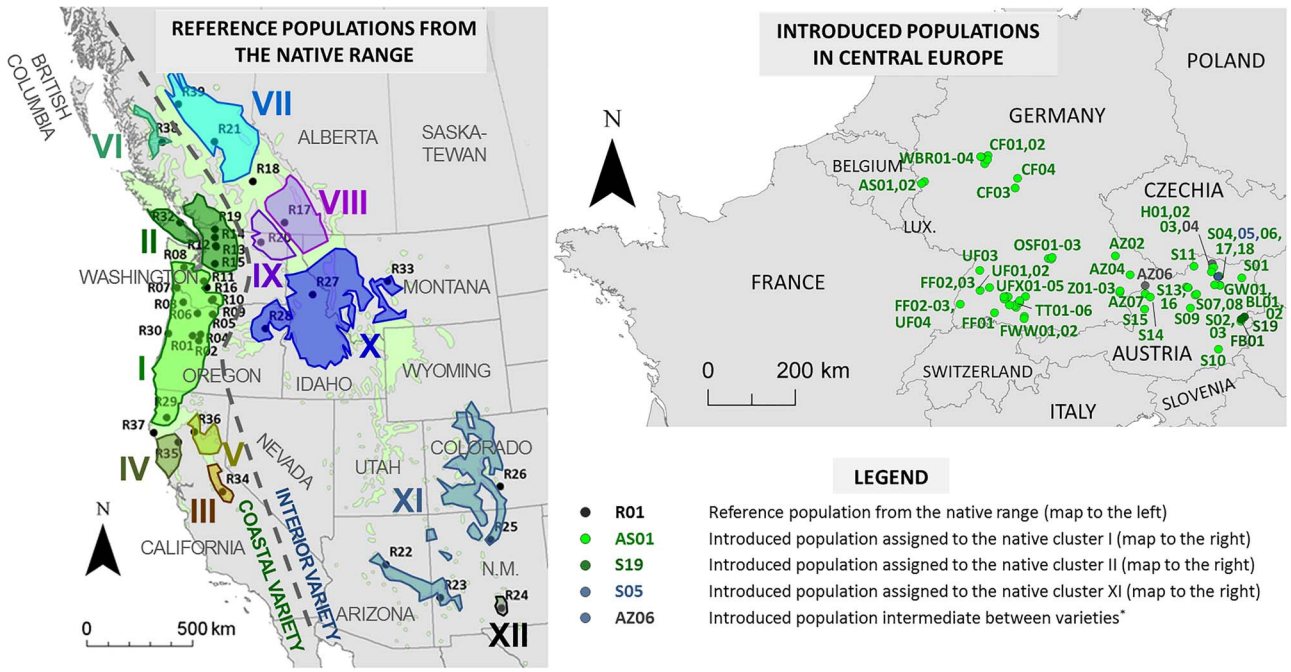

Figure 1 Location of study populations from the native range and introduced populations in Central Europe. The geographic distribution of different genetic clusters I–XII as described in Hintsteiner *et al.* (2018) is presented in the map of the native range (left). The assigned origin of introduced populations to native clusters is illustrated with different colours (right).

36 stands within which natural regeneration was present and (3) 36 juvenile populations from natural regeneration. Prior to population genetic analysis, we checked the genotypic data of the introduced adult populations for artificial admixture between the two varieties. We applied the method described in Hintsteiner *et al.* (2018) and performed a Bayesian clustering analysis using the STRUCTURE software (Falush *et al.*, 2003; Pritchard *et al.*, 2000), including all individuals from native and introduced populations.

We set a fixed number of two assumed clusters ($K = 2$), which correspond to the two varieties, coastal (*P. menziesii* var. *menziesii*) and interior *P. menziesii* var. *menziesii*, and performed 20 independent runs, employing 50 000 burn-in replications and 100 000 Markov chain Monte Carlo iterations, assuming admixture and correlated allele frequencies. We averaged individual membership proportions to each one of the two clusters and accounted for label switching by using the software CLUMPAK (Kopelman *et al.*, 2015), which implements the default algorithm

of the CLUMPP method (Jakobsson and Rosenberg, 2007). Where artificial intermixing of both varieties was supported by the Bayesian cluster analysis, we excluded from the dataset those trees that did not belong to the variety which was typical for the stand. We removed individuals assigned to the interior variety cluster (membership proportion to coastal variety <10 per cent, i.e. $q < 0.1$), which occurred within introduced populations assigned to the coastal variety cluster ($q > 0.9$) and vice versa. Subsequently, we applied the Microchecker software v. 2.2.3 and tested for the presence of null (non-amplified) alleles using the 'Brookfield 1' algorithm (Van Oosterhout *et al*., 2004). In case null alleles were supported by the statistical test, new genotypic tables were produced with corrected genotypes produced by the software according to the algorithm.

After preparing the dataset with the adjusted genotypes, we computed the mean number of alleles per locus, as well as the observed and expected heterozygosity, for each population using the software GenAlEx v. 6.5. In addition, given that the sample size varied among populations, we calculated the standardized allelic richness after rarefaction using the software ADZE (Szpiech *et al*., 2008). This measure is less sensitive to the variation of sample size (Petit *et al*., 1998). We chose 10 alleles as rarefaction size, which corresponded to the minimum number of genotyped individuals at a single locus within a single population. Next, we used population-wise diversity measures in order to make comparisons of genetic diversity levels between groups of populations. First, we compared genetic diversity between native and introduced populations and, second, between adult and juvenile populations in Central Europe. We performed two-sided Student's *t*-tests assuming equal variances in Excel (Microsoft). For the comparison between native and introduced adult populations of the coastal variety, we focused on the most common origin represented in our sample, which is located in an area between Central Washington and the border between Oregon and California, USA (Figure 1). In total, 62 out of 64 introduced adult populations show an origin from this area which includes 13 native populations (Cluster I, Hintsteiner *et al*. 2018). Thus, we compared the average diversity values between those 13 native and 62 introduced adult populations originating from this area.

Results

The adult Douglas-fir stands that we sampled in Central Europe exhibit similar levels of genetic diversity as native Douglas-fir populations in the region of North America where they originate from (Figure 2). In the native range, all measures of genetic diversity for the coastal variety reach their maximum in the area stretching between the border of California and Oregon in the South and the border between Washington and British Columbia in the North (Table 2). Cluster I, which covers the largest part of this area (Figure 1), displays the highest average number of alleles per locus ($A = 16.31 \pm 0.31$), observed ($H_o = 0.716 \pm 0.005$) and expected heterozygosity ($H_e = 0.903 \pm 0.002$) and standardized allelic richness ($AR_{10} = 7.50 \pm 0.05$) of all other native genetic clusters of the coastal variety (Table 2).

The European Douglas-fir populations which originate from this area (see cluster I) display similar diversity values of 16.84 ± 0.22 alleles per locus (A), an observed (H_o) and expected heterozygosity (H_e) of 0.691 ± 0.018 and 0.904 ± 0.001 respectively, and a standardized allelic richness (AR_{10}) of 7.53 ± 0.02 (Table 3). No significant differences were detectable between native populations belonging to cluster I and introduced (European) adult populations assigned to this cluster for any of the four genetic diversity parameters (Table 4).

Values of genetic diversity measures for each individual population are presented in Supplementary Data separately for native (Supplementary Table S1) and introduced populations (Supplementary Table S2).

Comparing the genetic diversity of our 36 adult European Douglas-fir stands versus juveniles resulting from natural regeneration within these stands revealed a reduction of genetic diversity for the natural regeneration. Adult trees showed an average of 16.76 ± 0.26 alleles per locus, an observed and expected heterozygosity of 0.703 ± 0.005 and 0.905 ± 0.001 respectively, and a standardized allelic richness of 7.52 ± 0.03 (Table 3). Three of these parameters had significantly lower values for the juveniles sampled in the regeneration (Figure 2; Tables 3 and 4). The observed heterozygosity did not differ significantly. Two of the 36 adult populations originate from cluster II (see Table 3). However, we did not treat them separately because clusters I and II do not exhibit large genetic differences.

Discussion

The high genetic diversity of adult Douglas-fir in Europe corresponds well with its native range in North America. Within this range, maximum genetic diversity levels have been revealed for the coastal Douglas-fir in Washington and Oregon based on allozymes (Li and Adams, 1989; Klumpp, 1999; Krutovsky *et al*., 2009), microsatellites (Krutovsky *et al*., 2009; Neophytou *et al*., 2016) and terpenes (Zavarin and Snajberk, 1973), and are confirmed by our study. This high genetic diversity of Douglas-fir has been attributed to the occurrence of large refugial populations that were less prone to genetic drift than small, peripheral refugia (Wei *et al*., 2011; van Loo *et al*., 2015). Large refugial populations, mainly around Willamette Valley and Puget, have been supported both by fossil evidence (Tsukada, 1982) and phylogenetic studies (Gugger *et al*., 2010; Gugger and Sugita, 2010; Wei *et al*., 2011). Therefore, the high genetic diversity found among mature trees of our study stands is likely the result of seed import from the aforementioned areas of the natural distribution range (Hintsteiner *et al*. 2018).

The introduction of non-native tree species may cause a reduction of genetic diversity due to population size bottlenecks (Dlugosch and Parker, 2008). Such effects are more pronounced if a limited amount of reproductive material is used for the establishment of introduced forest stands and/or if no repeated introductions take place (Lefèvre *et al*., 2004). This was obviously not the case for Douglas-fir in Central Europe. After a period of experimental plantations, the economic interest for Douglas-fir grew towards the end of 19th century (Lavender and Hermann, 2014). Shipment of large amounts of seeds from the native range began during this period and continued for decades (Wimmer, 1909; Podhorsky, 1927), ensuring a broad genetic base.

Table 2 Genetic diversity of native populations (mean and standard error). Subdivision into clusters within each variety follows Hintsteiner *et al.* (2018). *N* = number of populations; *A* = mean number of alleles per locus and population; H_o = mean observed heterozygosity over loci; H_e = mean expected heterozygosity per loci; AR_{10} = standardized allelic richness (rarefaction size = 10 alleles per population). The standard error is given where more than one population were analysed. The clusters within varieties are sorted from Northwest to Southeast. The group of populations used for a comparison with introduced populations is highlighted in bold letters.

Variety	*Cluster*	*N*	*A*	H_o	H_e	AR_{10}
Coastal (*P. menziesii* var. *menziesii*)	**VI**	**1**	**11.92**	**0.750**	**0.827**	**5.94**
	II	**6**	15.08 ± 0.37	0.710 ± 0.008	0.891 ± 0.004	7.22 ± 0.09
	I/II	**1**	**15.92**	**0.708**	**0.899**	**7.40**
	I	**13**	16.31 ± 0.31	0.716 ± 0.005	0.903 ± 0.002	7.50 ± 0.05
	R37*	**1**	**16.31**	**0.722**	**0.895**	**7.36**
	IV	**1**	**15.62**	**0.724**	**0.874**	**7.03**
	V	**1**	**13.38**	**0.759**	**0.851**	**6.45**
	III	**1**	**12.46**	**0.714**	**0.860**	**6.50**
	Overall	25	15.53 ± 0.30	0.718 ± 0.004	0.892 ± 0.004	7.26 ± 0.09
Interior (*P. menziesii* var. *glauca*)	**VII**	**2**	12.35 ± 0.95	0.710 ± 0.013	0.857 ± 0.012	6.57 ± 0.22
	VIII	**1**	**12.00**	**0.717**	**0.866**	**6.61**
	VIII/IX	**1**	**15.15**	**0.673**	**0.885**	**7.22**
	IX	**1**	**13.00**	**0.673**	**0.865**	**6.81**
	X	**3**	15.05 ± 0.62	0.654 ± 0.009	0.872 ± 0.005	7.15 ± 0.07
	VII–X	**8**	13.75 ± 0.62	0.681 ± 0.011	0.868 ± 0.005	6.90 ± 0.12
	XI**	**4**	14.90 ± 0.55	0.651 ± 0.022	0.817 ± 0.014	6.91 ± 0.13
	XII**	**1**	**13.60**	**0.648**	**0.787**	**6.68**
	XI–XII**	**5**	14.64 ± 0.50	0.65 ± 0.017	0.811 ± 0.013	6.86 ± 0.12

*Intermediate between clusters. The name of the population is given. More details on cluster assignment are given in Hintsteiner *et al.* (2018)
**Based on results from ten loci (loci PmOSU_2D6, PmOSU_3F1 and PmOSU_5A8 were removed due to lack of amplification).

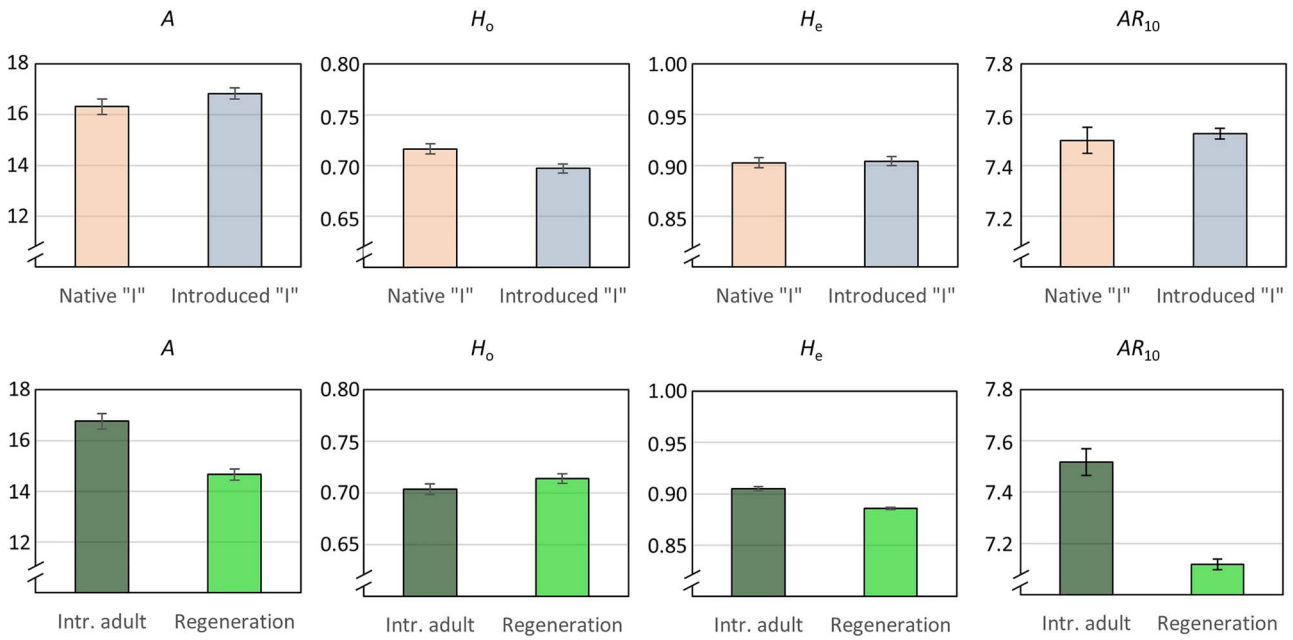

Figure 2 Comparison of genetic diversity: (a) between native populations of cluster I (see Figure 1 for its distribution in the native range) and native populations originating from this area of the native range (i.e. assigned to cluster I; Hinsteiner *et al.* 2018); (b) between adult trees and natural regeneration from 36 introduced stands (from Central Europe). *A* = mean number of alleles per locus and population; H_o = mean observed heterozygosity over loci; H_e = mean expected heterozygosity per loci; AR_{10} = standardized allelic richness (rarefaction size = 10 alleles per population). Error bars denote the standard error.

Table 3 Genetic diversity of introduced populations (sampled in Central Europe; mean and standard error). Assignment to clusters within each variety was made by Hintsteiner *et al.* (2018). *N* = number of populations; *A* = mean number of alleles per locus and population; H_o = mean observed heterozygosity over loci; H_e = mean expected heterozygosity per loci; AR_{10} = standardized allelic richness (rarefaction size = 10 alleles per population). The standard error is given where more than one population were analysed. The groups of populations used for pairwise comparisons are highlighted in bold letters

Variety	Cluster	Adult trees					Natural regeneration			
		N	*A*	H_o	H_e	AR_{10}	*A*	H_o	H_e	AR_{10}
Coastal (*P. menziesii* var. *menziesii*)	**I**[1]	34	16.75 ± 0.26	0.704 ± 0.010	0.905 ± 0.001	7.52 ± 0.03	14.50 ± 0.35	0.715 ± 0.01	0.885 ± 0.003	7.09 ± 0.05
	II[1]	2	16.85 ± 1.09	0.691 ± 0.018	0.904 ± 0.004	7.44 ± 0.05	17.31 ± 1.31	0.695 ± 0.001	0.908 ± 0.006	7.55 ± 0.16
	(I + II)[1]	**36**	**16.76 ± 0.26**	**0.703 ± 0.005**	**0.905 ± 0.001**	**7.52 ± 0.03**	**14.66 ± 0.36**	**0.714 ± 0.006**	**0.885 ± 0.003**	**7.12 ± 0.05**
	I[2]	28	16.94 ± 0.32	0.689 ± 0.006	0.904 ± 0.001	7.53 ± 0.02				
	Overall I	**62**	**16.84 ± 0.22**	**0.697 ± 0.004**	**0.904 ± 0.001**	**7.53 ± 0.02**				
	Overall I + II	64	16.84 ± 0.21	0.697 ± 0.004	0.904 ± 0.001	7.52 ± 0.02				
Interior (*P. menziesii* var. *glauca*)	**VII–IX**	2	14.80 ± 1.20	0.683 ± 0.000	0.893 ± 0.015	7.33 ± 0.43				
	XI *	1	15.30	0.708	0.836	6.63				

[1]Populations where both adult trees and regeneration were analysed.
[2]Populations where only adult trees were analysed.
*Based on results from ten loci (loci PmOSU_2D6, PmOSU_3F1 and PmOSU_5A8 were removed due to lack of amplification in some of the populations of the interior variety).

So far, only a few molecular genetic studies have dealt with the comparison of genetic diversity between native North American and introduced European Douglas-fir stands. None of the available studies indicated a reduction of genetic diversity after introduction (Hoffmann, 1994; Klumpp, 1999). Our study, based on a large dataset, provides further evidence that the introduction of Douglas-fir seed sources did not cause any population bottlenecks. Introduced stands planted with reproductive material imported from the native range are genetically as diverse as those growing in the native range.

However, we observed a significant reduction in the genetic diversity between old European Douglas-fir stands and the natural regeneration within these stands expressed by the mean number of alleles per locus (*A* and AR_{10}, respectively), and the expected heterozygosity (H_e), but not by the expected heterozygosity (H_o). This suggests a population bottleneck caused by a limited number of reproducing trees in the European populations. In particular, allelic richness (both *A* and AR_{10}) is more susceptible to drift caused by population bottlenecks than heterozygosity because it does not consider allele abundance, but only their presence or absence (Petit *et al.*, 1998; Greenbaum *et al.*, 2014). Given the high number of rare alleles, our microsatellite loci appear to be particularly sensitive and, thus, useful for assessing such bottleneck effects (Spencer *et al.*, 2000). Using isozymes, Konnert and Fussi (2012) did not detect any reduction of genetic diversity in the natural regeneration of German Douglas-fir stands. This might be due to the fact that these isozyme markers were significantly less polymorphic than our microsatellites (Konnert and Fussi, 2012). On the other hand, given the high polymorphism (>10 alleles per locus; see Tables 2 and 3), our sample sizes (around 20 individuals per site; see Table 1) may be inadequate for an accurate estimation of the observed heterozygosity in the population (Petit *et al.*, 1998).

Our results confirm previous studies revealing a significant reduction in the genetic diversity of seed lots from European Douglas-fir stands versus those from North America of similar origin (Eckhart *et al.* 2017). A recent study in German seed stands showed a significantly reduced genetic diversity in seed lots harvested from stands consisting of less than about 80 adult trees, while long-distance pollen flow could not compensate for the losses of genetic diversity (Wojacki *et al.*, 2019). Lack of phenological overlapping and strong fecundity differences may cause a further reduction of the effective population size (Zheng and Ennos, 1999; Fussi *et al.*, 2013; Korecký and El-Kassaby, 2016). Thus, a low number of reproducing trees seems to lead to a population bottleneck that evidently reduces the genetic diversity in the natural regeneration of Douglas-fir stands in Europe. Given the fact that the distribution of Douglas-fir in Europe is rather patchy compared to the regions of origin in North America (Kownatzki *et al.*, 2011; Wojacki *et al.*, 2019), bottleneck effects in the genetic diversity between the first and the following generations of European Douglas-fir are expected.

In addition to population bottlenecks, a small effective population size may also lead to increased assortative mating, selfing and, consequently, inbreeding depression (White *et al.*, 2007). Typically, increased inbreeding and selfing results in a deficit of heterozygotes at younger life stages, which is counterbalanced

Table 4 Results of pairwise two-sided *t*-tests (probabilities) between different groups of populations for different measures of genetic diversity: (a) between native populations of cluster I (see Figure 1 for its distribution in the native range) and native populations originating from this area of the native range (i.e. assigned to cluster I; Hinsteiner *et al*. 2018); (b) between adult trees and natural regeneration from 36 introduced stands (from Central Europe). *N* = number of populations; *A* = mean number of alleles per locus and population; H_o = mean observed heterozygosity over loci; H_e = mean expected heterozygosity per loci; AR_{10} = standardized allelic richness (rarefaction size = 10 alleles per population)

Diversity measure	Native (cluster I) *N* = 13	Introduced (cluster I) *N* = 62	*t*-Test	Introduced adult *N* = 36*	Introduced nat. Regeneration *N* = 36	*t*-Test
A	16.31± 0.31	16.84± 0.22	*P* = 0.301 (n.s.)	16.76± 0.26	14.66± 0.36	*P* < 0.001 ***
H_o	0.716 ± 0.005	0.697 ± 0.004	*P* = 0.061 (n.s.)	0.703 ± 0.005	0.714 ± 0.006	*P* = 0.183 (n.s.)
H_e	0.903 ± 0.002	0.904 ± 0.001	*P* = 0.548 (n.s.)	0.905 ± 0.001	0.885 ± 0.003	*P* < 0.001 ***
AR_{10}	7.50± 0.05	7.53± 0.02	*P* = 0.606 (n.s.)	7.53± 0.02	7.12± 0.05	*P* < 0.001 ***

*Includes only those populations where natural regeneration was sampled.

by natural selection against inbred individuals with increasing age (Yazdani *et al*., 1985; Marquardt and Epperson, 2004; Verbylaitė *et al*., 2017). This holds especially for conifer tree species which, in contrast to many angiosperms, lack self-incompatibility (White *et al*., 2007). Here, we did not observe a reduction of observed heterozygosity in the natural regeneration compared to mature trees, which may suggest that natural selection might have already eliminated inbred individuals.

Nevertheless, the risk of inbreeding depression in small and/or isolated introduced populations of Douglas-fir requires caution. A high percentage of empty seeds, pointing to inbreeding depression, has been observed in European Douglas-fir stands and was inversely proportional to the population size (Stauffer and Adams, 1993; Wojacki *et al*., 2019). What is more, inbreeding depression among others in survival and height growth has detrimental effects, not only in the early stages of seedling development but also in later life stages, up to mid-rotation age (Sorensen, 1999; Wang *et al*., 2004; Stoehr *et al*., 2015).

Conclusion

Mature Douglas-fir stands in Central Europe host a valuable gene pool characterized by both high levels of genetic variation and provenances with good growth performance. At the same time, maintenance of the genetic variation may be difficult if the reproducing population is small. This has the following implications for forest management practice:

1. If the old Douglas-fir stand population is limited and/or isolated, natural regeneration should be avoided to prevent a loss of genetic diversity.
2. Where natural regeneration is accepted, then an adequate number of seed trees should be used; taking advantage of several reproductive years is beneficial for the genetic diversity of natural regeneration. Moreover, supplementary planting or sowing can counterbalance the negative effects of natural regeneration where the size of the reproducing population is small (Valadon *et al*., 2010; Koskela *et al*., 2013).
3. The minimum population requirements for registration and harvesting of seed stands may need a revision to ensure the conservation of high genetic diversity in forest reproductive material. At present, a seed stand of Douglas-fir in Austria and Germany must cover a minimum of only 0.25 ha and must consist of a minimum of only 30 (in Austria) to 40 mature trees (in Germany), whereas the number of harvested trees for a commercial harvest can be as low as 10 (in Austria) to 20 (in Germany). As many as 100 reproducing trees may be required to avoid bottlenecks in the second and later generations of European Douglas-fir forests (Wojacki *et al*., 2019).
4. A possible solution is the use of seed orchards as seed sources because the number of clones and mating conditions there can be controlled to ensure a high genetic variation in the produced seeds. The high genetic diversity and suitable origin of adult Douglas-fir populations in Europe provide an excellent source for the selection of plus trees to be included in seed orchards. So far, large-scale breeding programs have been launched in France and Germany (Bastien *et al*., 2013; Liepe and Liesebach, 2018), to ensure high-quality seed sources for future generations of Douglas-fir forests in Europe.

Supplementary data

Supplementary data are available at *Forestry* online.

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