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Identification of varieties and gene flow in Douglas-fir exemplified in artificially established stands in Germany

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Abstract. Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] is an economically valuable non-native tree species in Germany and is considered very promising in view of global climate change. Therefore, the genetic characterization of Douglas-fir populations and seed stands in Germany is essential. We studied coastal and interior Douglas-fir varieties, both present in Germany, by using eleven isoenzyme and four microsatellite loci. By analyzing eight reference populations of known origin we were able to identify the two varieties on the population level using Bayesian and distance based methods. Seven populations present in Bavaria were then successfully assigned to one of the two varieties. Within varieties we found stronger grouping within the interior variety than within the coastal one. Despite lower differences within coastal Douglas-fir, we have first indications for the origin of two populations. For two Bavarian populations, natural regeneration was included and genetic data revealed no significant genetic difference between adults and offspring. The parentage analysis for one of the studied stands revealed that a large proportion of adults took part in the reproduction, but some trees were more successful than others in transferring their genes to the next generation. Our study was able to improve variety identification of Douglas-fir using isoenzyme markers and nuclear microsatellites and study reproductive patterns; both are important issues for the management of Douglas-fir stands in Bavaria.
Keywords *Pseudotsuga menziesii*, variety identification, coastal type, interior type, gene flow, nuclear microsatellites.

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Introduction

Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] is a native species in North America, ranging from South Canada down to Mexico and from the Pacific coast to the eastern slopes of the Rocky Mountains. Within this large area two varieties of Douglas-fir are distinguished by dendrologists: the coastal type (*Pseudotsuga menziesii* var. *menziesii*), also called "green Douglas-fir" and the interior type (*Pseudotsuga menziesii* var. *glauca*), also called "blue Douglas-fir". In Europe, a third type (*Pseudotsuga menziesii* var. *caesia*) called "grey Douglas-fir" has been described (Schenk 1939, Dallimore & Jackson 1966), but has not been accepted in general as a third variety in the literature. One explanation for the formation of the varieties is recolonization from different refugia at the Pacific coast and the Rocky Mountains after the Wisconsin ice age about 10,000 years ago (Halliday & Brown 1943). Recent studies on the phylogeography of Douglas-fir based on mitochondrial and chloroplast DNA connected the formation of Douglas-fir varieties with Pliocene orogeny more than 2 Ma (million) years ago and suggest three or more glacial refugia (Gugger et al. 2010).

Douglas-fir is an important tree species not only in North America, but has also been introduced in Europe more than 100 years ago. In Southern Germany, Douglas-fir of the coastal type has then been identified as a valuable non-native tree species for its high growth potential and good wood quality (e.g. Konnert & Ruetz 2006).

Since 1900 afforestation with Douglas-fir in Southern Germany has been increased. The origin of the material used in older stands is very often unknown. Some of the stands are of high quality. The trees were planted in groups and the number of trees within the stands sometimes varies strongly, from less than 20 up to a few hundreds. Based on morphological parameters (cone shape, foliage density) it is assumed that a mixture of coastal and interior Douglas-fir occurs to some extend within stands. Several of those stands were registered for seed harvest and started to naturally regenerate. Regardless of artificial or natural regeneration, forest owners want to know how to deal with such stands. In this context, it is important to know where those stands originate from, what the genetic diversity is and how genetic diversity is maintained from one generation to another.

Until recently, isoenzyme markers were used to analyze the native ranges of Douglas-fir in the USA and in Canada and differentiation of coastal (var. *menziesii*) and interior (var. *glauca*) type was found mainly based on two isoenzyme loci 6PGDH-A and PGM-A (Yeh & O'Malley 1980, El-Kassaby et al. 1982a, 1982b, Merkle & Adams 1987, Merkle et al. 1987). This method has been used to detect similarities between Douglas-fir plantations in Switzerland and seed stands in North America (Stauffer & Adams 1993), and for similar studies in several other European countries (Forrest et al. 2001, Fontes et al. 2003). Also in Germany, this method has been adopted for variety identification within planted stands of Douglas-fir (Hoffmann 1994, Hoffmann & Geburek 1995, Klumpp 1995, Leinemann 1996, 1998, Leinemann & Maurer 1999, Konnert et al. 2010). The development of nuclear microsatellite markers in Douglas-fir (Slavov et al. 2004, Krutovsky et al. 2009) offer new possibilities for a more precise classification of types and perhaps of provenances referring to a smaller geographical scale (e.g. seed zone).

In Germany, several provenance trials were established in order to compare growth and quality of different provenances on various sites distributed in Germany (e.g. IUFRO-provenance trial established 1970, Kleinschmit et al. 1991). The results from those long-term trials were utilized to provide recommendations for planting the best performing provenances of certain seed zones, depending on site conditions (Ruetz 1981). The seed material used for those provenance trials is of known origin. Therefore they provide very useful material,

not only to study differences in performance, but also to assess the genetic structure of the varieties.

The aims of the present study were: (i) to support the distinction between the coastal and the interior type of Douglas-fir based on isoenzymes with the additional application of nuclear microsatellite markers, (ii) to assess genetic variation of Douglas-fir populations planted in Southern Germany and try to trace them back to their region of provenance (seed zone) in North America and (iii) to determine the mating patterns using the example of a seed stand planted in Southern Germany.

Materials and methods

Plant material

Fifteen populations of Douglas-fir were studied (Table 1). Eight populations were of known origin and served as reference populations. Three of those populations represented seed lots from collections in 2010 in the USA (CR-WA, PF-WA and SR-WA). The seeds

Table 1 Description of analyzed plant material for 15 Douglas-fir populations of known and unknown origin.

Population Abbr.	Origin	Type	Location	Collective	Material	No. of individ.
CR-WA	Cispus River, WA (SZ430)	coastal	-	Seed lot	Embryo	88
PF-WA	Pack Forest, WA (SZ422)	coastal	-	Seed lot	Embryo	88
SR-WA	Sauk River, WA (SZ403)	coastal	-	Seed lot	Embryo	88
GR-WA	Grisdale, WA (SZ030)	coastal	Provenance trial – South Bavaria	Adult trees	Needles	33
ST-OR	Santiam River, OR (SZ462)	coastal	Provenance trial – South Bavaria	Adult trees	Needles	41
SA-BC	Salmon Arm, BC	interior	Provenance trial – Northwest Bavaria	Adult trees	Needles/Buds	35
SL-BC	Shuswap Lake, BC	interior	Provenance trial – Northwest Bavaria	Adult trees	Needles/Buds	27
PH-B	Snoqualmie, WA (SZ412)	coastal	Pegnitz-Hollfeld (Bavaria)	Young trees (20 years old)	Needles/Buds	96
FL-B	unknown	-	Flossenbürg (Bavaria)	Adult trees	Needles/Buds	40
GB-B	unknown	-	Griesbach (Bavaria)	Adult trees	Needles/Buds	52
FA-B	unknown	-	Frauenau (Bavaria)	Adult trees	Needles/Buds	38
SC-A-B	unknown	-	Schnaittenbach (Bavaria)	Adult trees	Needles/Buds	53
SC-NR-B	unknown	-	Schnaittenbach (Bavaria)	Natural regeneration	Needles/Buds	96
FR-A-B	unknown	-	Freising (Bavaria)	Adult trees	Needles/Buds	142
FR-NR-B	unknown	-	Freising (Bavaria)	Natural regeneration	Needles/Buds	245
Total						1162

Note. Needles and buds were used for nuclear microsatellite and isoenzyme analysis, respectively. Abbreviations: WA, Washington (USA); OR, Oregon (USA); BC, British Columbia (Canada); B, Bavaria (Germany); SZ, seed zone.

were harvested by the SILVA SEED company in OECD-certified stands, belonging to the coastal Douglas-fir (var. *menziesii*). Furthermore, sampling comprised of four Douglas-fir provenances growing in two provenance trials in Southern Germany (Elsendorf/Freising and Stadtsteinach/Nordhalben). The provenance trial "Elsendorf/Freising", established in 1980 with different provenances derived from natural forests in British Columbia (Canada), Washington (USA) and Oregon (USA). The provenances originating from Grisdale (GR-WA) and Santiam River (ST-OR) were collected from this provenance trial and belong to the coastal Douglas-fir. Two provenances were sampled from the provenance trial "Stadtsteinach/Nordhalben", established in 1978: Salmon Arm (SA-BC) and Shuswap Lake (SL-BC), originating in British Columbia (Canada) and belonging to the interior type (var. *glauca*). One additional reference population of coastal Douglas-fir was sampled in "Pegnitz-Hollfeld" (PH-B), which is a 20 years old stand established with seed material from the Snoqualmie region (Washington, USA).

Five stands planted in Southern Germany (Bavaria) at the beginning of the 20th century (FL-B, GB-B, FA-B, FR-A-B, SC-A-B) are of unknown origin. For two of those stands, the natural regeneration was also included in the study (FR-NR-B and SC-NR-B).

Within the gene flow study plot "Freising", all adult Douglas-fir trees were sampled. Altogether, 142 adults (FR-A-B) and 245 young trees from natural regeneration (FR-NR-B) were analyzed (Table 1). The stand comprised five groups of Douglas fir trees (AI-AV, Fig. 1). Adult trees are marked by triangles and plots are numbered by roman numerals. The natural regeneration for each plot is illustrated by dots and numbers are given next to the plots. All young trees higher than 20 cm within the plot were sampled. This is the general starting height for counting seedlings in forest inventory.

For determining the genetic structure, the

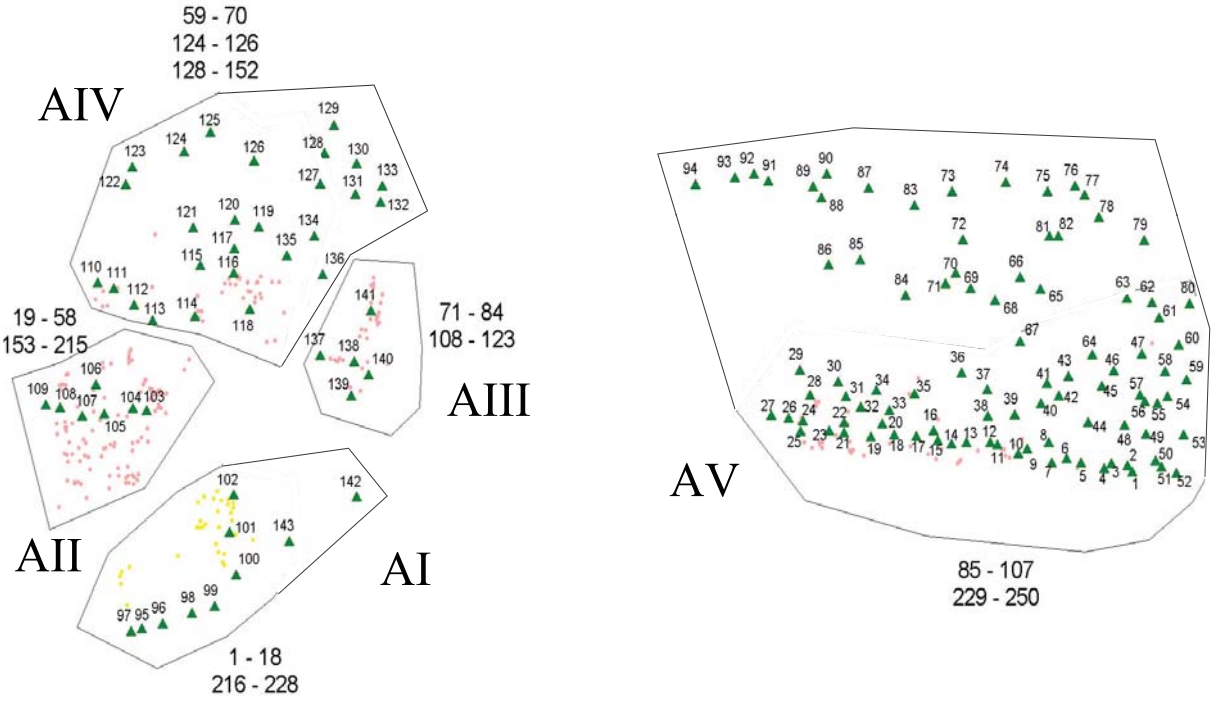

Figure 1 Gene flow study plot "Freising" (FR-A-B and FR-NR-B). Plots AI-AV are distinguished by roman numerals. Adult trees are represented by triangles and numbers. Natural regeneration is marked by dots and the numbers of trees sampled are given next to the plots. Subpopulation 1 comprises plots AI-AIV and subpopulation 2 equals plot AV

five plots were compared as such and also combined into two subpopulations, with the first one comprising plots AI-AIV (48 adults, 201 offspring) and the second one representing plot AV (94 adults, 44 offspring). The distance between the two subpopulations was about 50 m. The position of adults and saplings was determined by GPS, using the TOPCON system. To improve the accuracy of the measurements, the raw data were corrected by using a fixed point of known position. Thus, the measuring error was reduced to 2-3 m.

Genetic analysis

The number of analyzed individuals per population is given in Table 1, for isoenzymes and nuclear microsatellites. Because of missing dormant buds, isoenzyme analysis were not performed for two populations (GR-WA, ST-OR), thus only for 13 populations isoenzyme data was available. The following 11 isoenzyme loci representing 7 enzyme systems were used: AAT-A, AAT-B, AAT-C, FEST-B, IDH-A, MDH-A, MDH-B, MDH-C, PGI-B, PGM-A, 6PGDH-A. The distinction of varieties was based on allele frequencies at the loci 6PGDH-A and PGM-A. The isoenzyme analysis was performed using starch gel electrophoresis and procedures used by Konnert (2004).

DNA was extracted for 15 populations using the ATMAP method (Dumolin et al. 1995). Seeds were germinated and 5 mg of the emerging seedlings and 10 mg needles from adults and natural regeneration were freeze-dried (lyophilized) for 24 hours. DNA extracts were fluorometrically quantified (Gene Quant Pro, Amersham Bioscience) and adjusted to 20 ng/µL, or approximately diluted according to visual quantification on agarose gels. Altogether, 1162 individuals were genotyped at four highly polymorphic nuclear microsatellite loci PmOSU_2G12, PmOSU_3B2, PmOSU_3G9, and PmOSU_4A7 (Slavov et al. 2004, Krutovsky et al. 2009), fluorescently labelled by Cy5, IRD700, Cy5 and IRD700, respectively. A Multiplex PCR reaction was performed using fluorescent labelled primers in a mixture of 10 µl total volume containing 1 X reaction buffer (Qiagen), 0.2 µM of each primer and about 20 ng template DNA. The program for amplification was optimized using gradient PCR-conditions. The final PCR program started with initial denaturation at 95 °C for 15 min, followed by 30 cycles of 94 °C for 30 sec, 54 °C for 90 sec, 72 °C for 30 sec and a final elongation step at 60 °C for 30 min. The length of the PCR fragments was determined by using an automated sequencer (CEQ8000 Beckman-Coulter) and analyzed by using an internal size standard. Fragment length determination and allele assignment were carried out using the fragment analysis tool of CEQ8000 (Beckman-Coulter).

Data analysis

For isoenzymes and nuclear microsatellites GenAlEx 6.41 (Peakall & Smouse 2006) was used to calculate the mean number of alleles (N_A), gene diversity (N_E for the effective number of alleles, Brown & Weir 1983), observed heterozygosity (H_o, Hartl & Clark 1997), expected heterozygosity (H_E, Hartl & Clark 1997) and the inbreeding coefficient (F_{IS}, Hartl & Clark 1997). By using the FSTAT software (Goudet 1995), inbreeding coefficients (F_{IS}) for each locus and population was computed and significance was tested by performing 10,000 randomizations of alleles among individuals within samples. For nuclear microsatellites, possible scoring errors and occurrence of null (non-amplifiable) alleles were checked by using the software Microchecker (Van Oosterhout et al. 2004). Based on this software, scoring errors, like large allele drop-off (poor amplification of large-sized alleles) and error due to stuttering, both leading to homozygote excess, were proved. In order to detect deviations from Hardy-Weinberg-equilibrium due to null alleles, the software conducts simulations within each population. Instructions within

the user guide were followed and Brookfield1 measure was used to correct for null alleles. Brookfield1 equation accounts for null-allele homozygotes degraded DNA or problems with the PCR. The adjusted frequencies accounting for null alleles were then used for further statistical analysis.

Correlation was calculated for diversity measures (N_A, N_E, H_o, H_E and F_{IS}) between isoenzymes and nuclear microsatellites for all populations. Therefor a linear regression model in R (R Core team 2013) was used.

The software GenAlEx 6.41 (Peakall & Smouse 2006) was also used to estimate genetic distance between populations following Nei (1972). A principal coordinate analysis (PCoA) was performed to group populations according to their geographical origin based on pairwise F_{ST} values (Hartl & Clark 1997) using GenAlEx 6.41 again (Peakall & Smouse 2006). A Bayesian approach (STRUCTURE, Falush et al. 2003) was used to identify two varieties of Douglas-fir. The analysis parameters were set to 20,000 runs in the burn-in period, then 20,000 iterations, under the admixture model. K was set to 1-15 with 20 iterations per *K*. The highest "Delta K" corresponds to the uppermost level of hierarchy among runs with K = 1-15 based on the rate of change between successive *K* values (Evanno et al. 2005). This procedure was followed for isoenzymes, nuclear microsatellites and the combination of both datasets.

Again the software GenAlEx 6.41 (Peakall & Smouse 2006) was used to perform a PCoA for illustrating pairwise F_{ST}s for the study plot "FR-A-B" (a) between subpopulation 1 and subpopulation 2 and (b) between five plots for adults (AI-AV) and natural regeneration (NRI-NRV).

A parentage analysis was performed for the 245 offsprings within "FR-A-B" study site, using the software CERVUS 3.0 (Marshall et al. 1998, Kalinowski et al. 2007). To identify parent-pairs (sexes unknown) for each offspring all candidate parent genotypes on the site were used as parental information. An error rate of 0.01 was used, as suggested in the user manual and by Slavov et al. (2005b).

Results

Differentiation between varieties

As a first step, we used four reference populations of coastal Douglas-fir (CR-WA, SR-WA, SR-WA and PH-B) and two reference populations from British Columbia (SA-BC and SL-BC), representing interior Douglas-fir, to compare isoenzymes and nuclear microsatellites for their ability to distinguish between varieties. The results show that four loci, two isoenzyme and two microsatellite loci, are the most informative for differentiation between the varieties, because of allele frequency differences (Table 2). Populations representing coastal Douglas-fir were characterized by lower proportions of 6-PGDH-A6 (2-6%), compared to populations representing the interior Douglas-fir (54-67%). Furthermore, coastal Douglas-fir populations showed smaller amount of PGM-A6 (3-9%) than interior Douglas-fir populations (17-33%). In addition, two of the microsatellite markers (PmOSU_3B2, PmOSU_4A7) characterized by the highest F_{ST} values (0.037 and 0.032, respectively) were most efficient in distinguishing between the two varieties. The allele 96 at locus PmOSU_3B2 and the allele 242 at locus PmOSU_4A7 had high proportions in the interior Douglas-fir populations, whereas coastal Douglas-fir populations had very low proportions/absence of the mentioned "interior" alleles (Table 2). Following the allele frequencies at the four mentioned loci, populations of unknown origin can be identified as coastal or interior Douglas-fir. Thus, population SC-A-B and its natural regeneration SC-NR-B were attributed to the interior type, whereas populations GB-B, FA-B, FR-A-B and FR-NR-B were identified as the coastal type (allele frequency proportions not

Table 2 Allele frequencies for coastal (CR-WA, PF-WA, SR-WA, PH-B) and interior (SA-BC and SL-BC) Douglas-fir populations exemplified at the most discriminative markers: two isoenzyme (6-PGDH, PGM) and two nuclear microsatellite loci (PmOSU_3B2, PmOSU_4A7)

	CR-WA	PF-WA	SR-WA	PH-B	SA-BC	SL-BC
6-PGDH						
A3	0.91	0.91	0.92	0.92	0.43	0.28
A6	0.06	0.03	0.06	0.06	0.54	0.67
all others (2 alleles)	0.03	0.05	0.02	0.02	0.03	0.06
PGM						
A4	0.79	0.84	0.86	0.81	0.50	0.65
A6	0.09	0.03	0.05	0.08	0.33	0.17
all others (4 alleles)	0.12	0.13	0.10	0.11	0.17	0.19
PmOSU_3B2						
Allel 96	0.06	0.03	0.02	0.05	0.23	0.22
all others (68 alleles)	0.94	0.97	0.98	0.95	0.77	0.78
PmOSU_4A7						
Allel 242	0.00	0.01	0.01	0.02	0.14	0.15
all others (68 alleles)	1.00	0.99	0.99	0.98	0.86	0.85

shown). For population FL-B, showing intermediate F_{ST} -values, we assume mixed material of coastal and interior Douglas fir.

The rate of change of posterior probability between successive runs was highest for K = 2 for isoenzymes, nuclear microsatellites and the combined dataset (in Fig. 2a, results for the combined dataset). Two subpopulations are presented, corresponding to the two varieties. Subsequently, the populations were attributed to the two varieties, by applying the proportion of membership of each population of two gene pools for each population. Figure 2b illustrates the results, by assuming two gene pools (cluster 1 and 2) for each of the populations, based on 11 isoenzyme markers. Cluster 1 represents the interior Douglas-fir, whereas cluster 2 represents the coastal type. Populations originating from the interior region had more than 60% of the interior cluster 1. Populations of the coastal type showed less than 50% of cluster 1. Figure 2c shows the proportion of membership of the two gene pools for each of the populations, based on four microsatellite markers. Populations SA-BC and SL-BC carry high proportions of "interior alleles". Both populations originated from the distribution area of interior Douglas-fir. Moreover, population SC-A-B, together with its natural regeneration (SC-NR-B), as well as population FL-B from Bavaria, showed high proportions of cluster 1. All other populations displayed less than 50% membership to the interior gene pool.

Using the combined dataset of isoenzymes and nuclear microsatellites, the proportion of the interior gene pool (cluster 1) decreased below 33% for coastal Douglas fir populations. Interior Douglas-fir populations displayed (Fig. 2d) more than 80% of the interior Douglas-fir gene pool, except for population FL-B, showing around 60%. This result for FL-B was consistent for isoenzymes (Fig. 2b), nuclear microsatellites (Fig. 2c) and the combined dataset (Fig. 2d) and might hint to the use of mixed material for the establishment of the stand. Summarizing STRUCTURE results, the proportion of membership for isoenzymes showed the lowest distinction, results for nuclear microsatellites were more distinctive and results based on the combined dataset showed the best distinction of varieties.

Genetic variation within populations

For the 13 populations analyzed with isoenzymes, the mean number of alleles (N_A) varied between 1.2 and 3.2 and the effective number of alleles (N_E) between 1.16 and 1.33 (Table

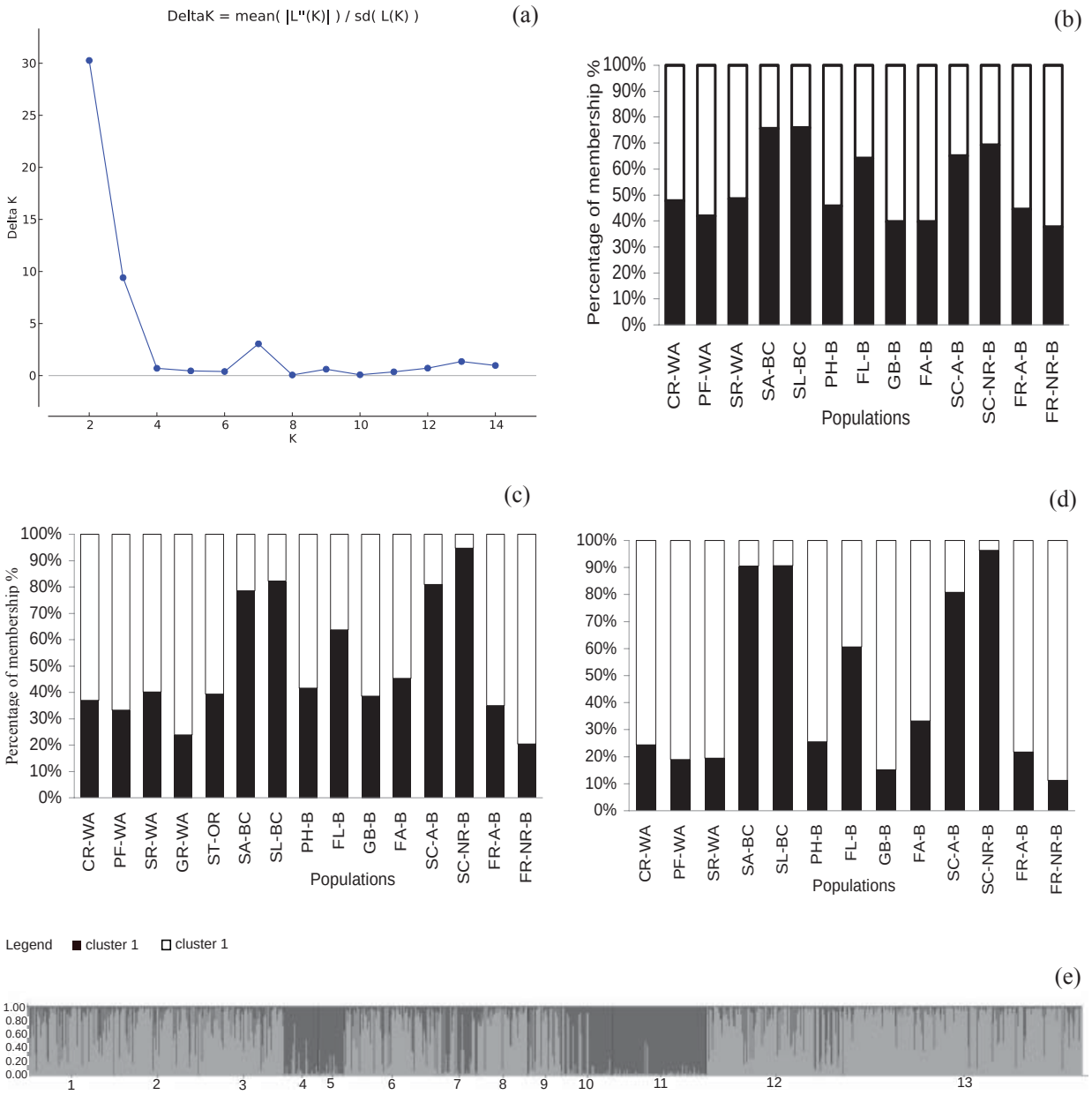

Figure 2 Results for STRUCTURE analysis: (a) values of the statistic "Delta K" for all calculated K-values from 1-15 based on the combined dataset of isoenzymes and nuclear microsatellites. The highest "Delta K" corresponds to the uppermost level of hierarchy among runs (Evanno et al. 2005), (b) proportion of membership obtained with the software STRUCTURE at $K = 2$ of each population in two gene pools based on 11 isoenzyme loci (c) based on four nuclear microsatellites and (d) based on the combined dataset of nuclear microsatellites and isoenzymes (e) proportion of membership of each individual to two assumed subpopulations ($K = 2$). Cluster 1 in dark grey represents the interior and cluster 2 in light grey the coastal Douglas-fir variety

3). The observed (H_O) and expected (H_E) heterozygosity ranged from 0.119 to 0.188 and from 0.129 to 0.190, respectively, resulting in inbreeding coefficients (F_{IS}) varying from -0.073 to 0.181. The values were non-significant within the analyzed populations, except for SL-BC, where a significant inbreeding coefficient was detected (Table 3).

Table 3 Summary of means of diversity values for isoenzymes (ISO) and nuclear microsatellites (SSR) over all loci for each of the 13 and 15 analyzed populations, respectively

	N_A		N_E		H_O		H_E		F_{IS}	
Pop	ISO	SSR	ISO	SSR	ISO	SSR	ISO	SSR	ISO	SSR
CR-WA	3.2	27.8	1.25	14.4	0.164	0.734	0.183	0.902	0.108 n.s.	0.191**
PF-WA	2.9	30.5	1.21	15.8	0.147	0.755	0.160	0.919	0.082 n.s.	0.184**
SR-WA	2.9	26.5	1.16	13.4	0.126	0.758	0.132	0.904	0.049 n.s.	0.167**
GR-WA	-	19.3	-	11.7	-	0.826	-	0.897	-	0.094*
ST-OR	-	24.0	-	14.6	-	0.793	-	0.911	-	0.139**
SA-BC	2.3	19.5	1.33	11.2	0.156	0.765	0.166	0.901	0.074 n.s.	0.153**
SL-BC	2.1	17.5	1.25	10.9	0.121	0.733	0.147	0.903	0.181*	0.202**
PH-B	3.1	25.8	1.22	14.6	0.160	0.823	0.160	0.917	0.007 n.s.	0.106**
FL-B	1.3	24.3	1.30	13.2	0.183	0.710	0.190	0.908	0.051 n.s.	0.229**
GB-B	1.2	19.5	1.16	10.2	0.119	0.728	0.129	0.885	0.085 n.s.	0.188**
FA-B	2.5	24.0	1.20	13.8	0.161	0.873	0.153	0.924	-0.040 n.s.	0.069*
SC-A-B	2.6	20.0	1.29	9.1	0.188	0.760	0.174	0.881	-0.073 n.s.	0.146**
SC-NR-B	2.6	18.0	1.31	7.2	0.176	0.762	0.178	0.856	0.016 n.s.	0.116**
FR-A-B	3.1	32.3	1.23	16.3	0.167	0.740	0.174	0.923	0.042 n.s.	0.202**
FR-NR-B	3.1	31.0	1.24	14.5	0.171	0.748	0.171	0.909	-0.000 n.s.	0.179**
Mean	2.7	24.6	1.30	12.9	0.157	0.775	0.116	0.905	0.059	0.145

Note. N_A - mean number of alleles, N_E - effective number of alleles, H_O - observed heterozygosity, H_E - expected heterozygosity, F_{IS} - inbreeding coefficient. Significance of F_{IS} values: non-significant (n.s.), *$p < 0.01$, **$p < 0.001$

For all 15 populations, analyzed with nuclear microsatellites, the mean number of alleles (N_A) was higher compared to the isoenzymes and varied between 17.5 and 32.3 and the effective number of alleles (N_E) between 7.2 and 16.3 (Table 3). The highest N_A was found in population FR-A-B with the highest number of analyzed trees. Generally, low N_A values were found in two populations from provenance trials with relatively low numbers of analyzed individuals. Nevertheless, no clear linear relationship between N_A and the number of analyzed individuals per population was detected. For example, population ST-OR with 41 sampled individuals had a higher value for N_A (24.0) than population SC-NR-B with 96 sampled individuals (18.0). A closer inspection of the dataset revealed that interior Douglas-fir populations had generally less alleles than coastal Douglas-fir. This trend was shown less clearly also for N_E, which is a value less dependent on the sample size.

The observed (H_O) and expected (H_E) heterozygosity ranged from 0.710 to 0.873 and from 0.856 to 0.924, respectively, resulting in significantly high inbreeding coefficients (F_{IS}) varying from 0.094 to 0.229 for each population over all loci (Table 3). Those values were already corrected for null alleles based on estimations calculated with Microchecker. The still high values of F_{IS} were surprising, because based on isoenzymes no departure from HWE was found, except for population SL-BC. Therefore, we calculated F_{IS} values for each locus separately within populations (Table S1) leading to significant results for Locus PmOSU_4A7 in all populations (range 0.101 to 0.386). For locus PmOSU_3G9 all populations except three (FA-B, GR-WA, ST-OR) showed significant deviation from HWE (range -0.39 to 0.448). Locus PmOSU_3B2 showed significant F_{IS} in all but four populations (SC-A-

B, SC-NR-B, ST-OR, SA-BC) as well (range 0.039 to 0.195). PmOSU_2G12 showed significant F_{IS} values only in 8 populations (range for all populations -0.009 to 0.194).

A comparison of isoenzyme and nuclear microsatellite diversity values for each population revealed slight, but significant correlations for N_A and F_{IS}-values ($r^2 = 0.29$, $p < 0.05$ and $r^2 = 0.28$, $p < 0.05$, respectively). For the other diversity measures no correlation was detected. This was supposed given the different genetic background of the two marker systems.

Diversity measures were also inspected for their ability to distinguish between varieties. Concerning diversity measures isoenzymes exhibited higher N_E-values for the interior type, whereas nuclear microsatellites had higher diversity values for coastal Douglas-fir (Table 3). For the other diversity measures no strong trends for identifying Douglas fir varieties were found.

Genetic differentiation between populations

Results for genetic differentiation (Nei 1972) between populations calculated for isoenzymes and microsatellites separately and combined for both datasets are given in Table 4. Calculations for isoenzymes resulted in genetic distances between all populations except natural regeneration from 0.001 to 0.104. Within coastal Douglas-fir genetic distances were lower (0.001-0.004) than within interior Douglas-fir (0.007-0.096). The highest genetic distances were found for coastal vs. interior Douglas-fir populations (0.010-0.104). For nuclear microsatellites, calculations for all populations revealed higher values compared to isoenzymes ranging from 0.096 to 0.789. The highest values were found for coastal vs. interior Douglas-fir populations (0.249-0.789). For the combined dataset, again lower values and thus lower differentiation between populations were detected. Highest discrimination within Douglas coastal fir was calculated based on microsatellites.

For identification of groups of populations, we used PCoAs based on pairwise F_{ST} genetic distances. Figure 3a includes all the populations and the first two coordinates explained 65% of the total variation. Coordinate 1 indicates the separation of coastal and interior Douglas-fir. Five populations (SC-A-B, SC-NR-B, SA-BC, SL-BC and FL-B) were separated from the others. Those five populations have already been classified as interior Douglas-fir by the STRUCTURE analysis.

Higher among population differentiation was detected for the interior Douglas-fir, compared to the coastal one. SC-A-B had the highest genetic distance, whereas the populations from British Columbia (SA-BC, SL-BC) were

Table 4 Comparison of Nei's genetic distance between 11 and 13 Douglas-fir populations using 11 isoenzyme (ISO) and four microsatellite (SSR) markers, respectively. Minimum (min) and maximum (max) distance values are shown between populations within coastal and interior Douglas-fir and between coastal and interior Douglas-fir populations. Nei's genetic distance for adults and natural regeneration of two Bavarian Douglas-fir populations are given separately

	ISO		SSR		SSR+ISO	
Varieties	min.	max.	min.	max.	min.	max.
coastal	0.001	0.004	0.096	0.415	0.004	0.016
interior	0.007	0.096	0.251	0.467	0.017	0.110
coastal - interior	0.010	0.104	0.249	0.789	0.026	0.122
Adults - offspring						
FR-A-B - FR-NR-B	0.002		0.089		0.005	
SC-A-B - SC-NR-B	0.002		0.063		0.005	

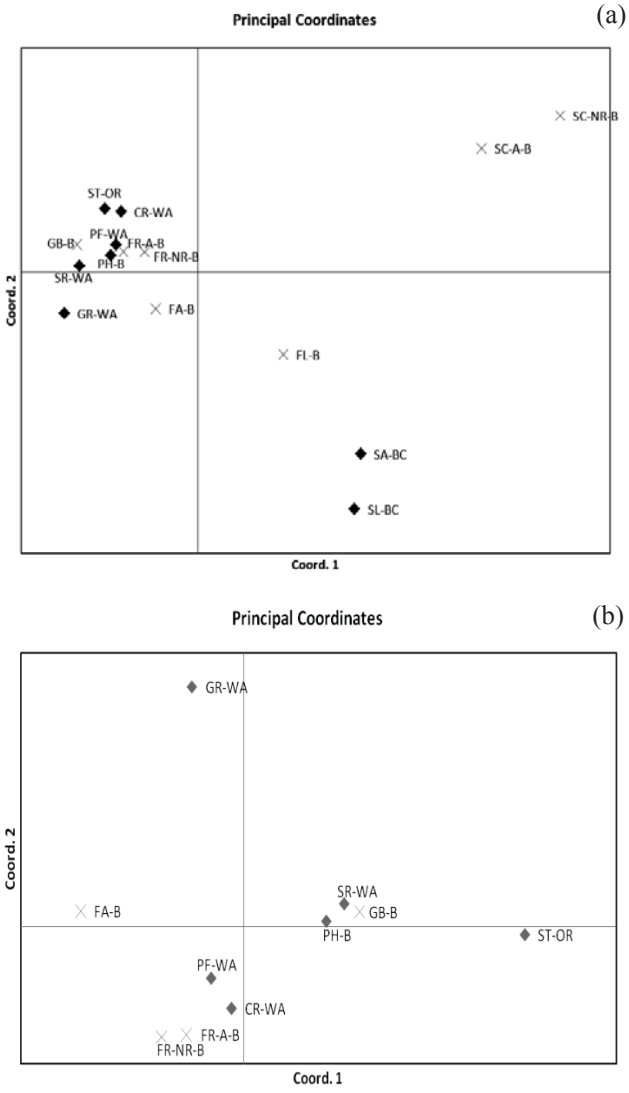

Figure 3 Principal coordinate analysis (PCoA) of pairwise F_{ST} of (a) coastal and interior Douglas-fir populations and (b) coastal Douglas-fir populations based on nuclear microsatellites. Diamonds represent populations of known origin and crosses represent populations of unknown origin

grouped (Fig. 3a). Among populations from the coastal Douglas-fir, genetic differentiation is less pronounced. Certain groups could be identified and are visualized in the PCoA analysis (Fig. 3b). One group was formed by the populations SR-WA and PH-B, originating from Northern Washington (seed zones 403 and 412). A second group was formed by populations south of Mt. Rainier (PF-WA and CR-WA, seed zones 422 and 430, respectively). The population GB-B was more similar to the first group of populations from Northern Washington, whereas the population FR-A-B was grouped with the second group of reference populations. The population GR-WA (Olympic Peninsula, seed zone 030) from Washington and the more southern population from Oregon (ST-OR, seed zone 462) did not group with other analyzed populations. The population FA-B could not be assigned to any of the analyzed reference populations.

Genetic characterization and gene flow within Bavarian Douglas-fir seed stands

For gene flow studies, we used the four selected nuclear microsatellites, because of their high level of polymorphism, compared to isoenzymes. Both stands, where adults and offspring were analyzed (FR-A-B, SC-A-B), revealed lower values for the number of alleles (N_A) and genetic diversity (N_E) within the offspring (Table 3). Nei's genetic distance between adults and their natural regeneration was low in both stands, but slightly higher in FR-A-B compared to SC-A-B based on microsatellites (Table 4).

Within the stand FR-A-B, two subpopulations were delineated and genetic diversity was calculated separately for adults and natural regeneration for each subpopulation. N_E for adults (A_subpopulation 1) and natural regeneration within subpopulation 1 (NR_subpopulation 1) was 15.9 and 14.1, respectively. N_E for adults and natural regeneration of subpopulation 2 was 15.1 and 12.3, respectively. The genetic distances between subpopulation 1 and subpopulation 2 were displayed in a PCoA (Fig. 4a), where the first two coordinates explained 79.7% of the variation. The PCoA showed separation of all four groups. The lowest distance was detected between adults and natural regeneration from subpopulation 2. Unexpected high genetic dis-

tance was observed between adults and natural regeneration from subpopulation 1. The two subpopulations were further divided into five plots (Fig. 1). The PCoA displayed the genetic distance between single plots (Fig. 4b), with the first two coordinates explaining 61% of the variation. When comparing single plots, genetic distance between some of them was high, possibly influenced by low sample numbers in plot AII and AIII (Fig. 4b). Adults from plot AI, AII and AIII were clearly different from the adults of plot AIV and AV. Also natural regeneration patterns were against expectations, because the analyzed offspring was most similar to the adults from plot AIV and AV.

Performing a parentage analysis, 174 offspring (71%) could be assigned to a parent pair with 95% confidence, while 188 offspring (77%) could be assigned if a lower confidence level of 80% was accepted. For further interpretation parentages assigned with 95% were used. The four loci used in this study displayed high polymorphic information content (PIC) ranging from 0.848 to 0.966. In total, 108 adult trees (76%) contributed to the analyzed natural regeneration (Fig. 5). Tree number 107 (plot AII) was most successful with the highest parentage percentage of 18.5 % (91 times out of 2x245). Sixteen trees had parentage percentages of more than 5%.

The percentage of young trees derived from adult trees of certain plots can be found in Table 5. Here, detailed information on reproductive patterns of each plot is available, showing that in general adults from plot AV contributed the most to the natural regeneration of all other

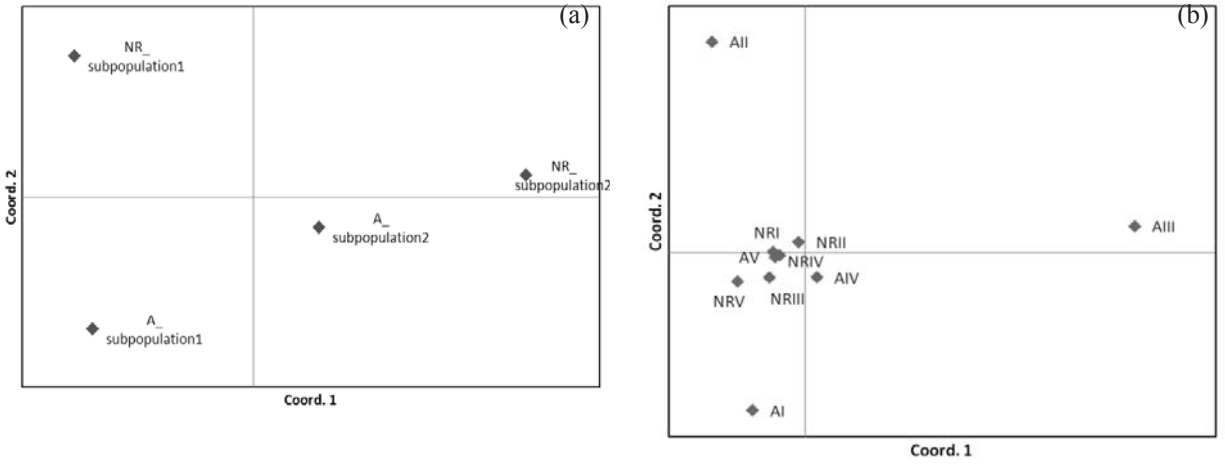

Figure 4 Principal coordinate analysis (PCoA) of pairwise F_{ST} values of adults (A) and natural regeneration (NR) within population FR-A-B for (a) between two subpopulations and (b) between five plots

Table 5 Proportion of offspring (NR) derived from parents (A) of five different plots within population FR-A-B

	NRI	NRII	NRIII	NRIV	NRV	No. adults
AI	0.08	0.11	0.14	0.10	0.09	10
AII	0.11	0.11	0.16	0.09	0.00	7
AIII	0.00	0.03	0.00	0.03	0.00	5
AIV	0.11	0.12	0.14	0.20	0.13	26
AV	0.48	0.43	0.40	0.35	0.64	94
Total	0.79	0.81	0.83	0.76	0.85	142
External gene flow	0.21	0.19	0.17	0.24	0.15	
No. offspring	31	101	29	40	44	245

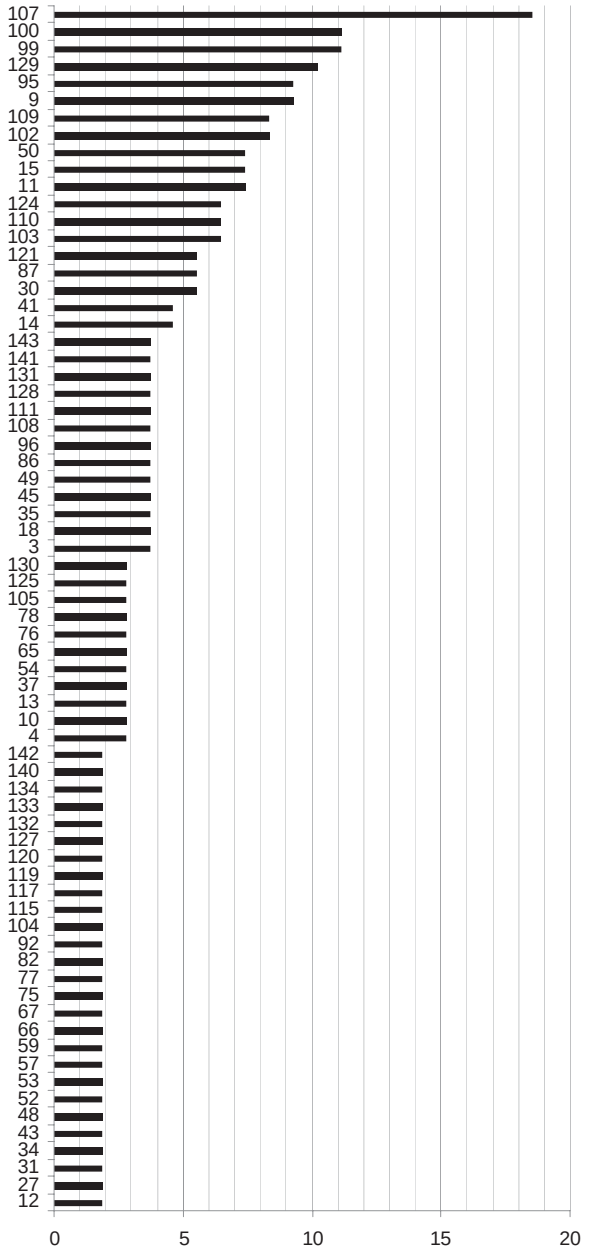

Figure 5 Distribution of parentage (%) to the offspring of population FR-A-B, based on four highly polymorphic nuclear microsatellite markers. Tree numbers are given at the side of the graph. Parents participating only once in the reproduction are not displayed in the figure

plots. In plot AV, the natural regeneration represented the genetic structure of the parents. The majority of the natural regeneration of the other four plots was also derived from adults of plot AV. Adults of plot AII contributed only up to 16% and adults of plot AIII contributed scarcely (3%) to the natural regeneration of each plot, probably caused by the low number of adults within these plots. The contribution of adults from outside the stand to the natural regeneration was distributed evenly in all plots, ranging from 15 to 24%. The sometimes high values of gene flow from outside the stand can be explained by pollen and seed flow from stands located at a distance of 300 – 400 m near the study plot.

Discussion

The results of our study provide useful genetic tools for the identification of the two Douglas-fir varieties and give insights into reproductive processes and their consequences on the genetic structures of the natural regeneration of Douglas-fir stands. One of the main objectives of this study was to improve the genetic differentiation approach between coastal and interior Douglas-fir. If variety distinction based on genetic markers can be improved, the origin of the forest reproductive material (Douglas-fir seeds) and, therefore the genetic quality, can be surveyed more precisely. This is a crucial issue for forest practice, since there is a great variety dependent variation in growth performance of Douglas-fir in Europe, with the coastal type as the preferred variety (Kleinschmit et al. 1991). Moreover, until recently, the provenance documentation of imported seed material was often missing. Thus, a reliable method for variety identification is essential.

Starting from work in America (Krutovsky et al. 2009), our study is the first one using both isoenzyme and nuclear microsatellite markers to genetically characterize planted Douglas-fir stands in Europe. Based on material of known origin, we found two microsatellite markers (PmOSU_3B2, PmOSU_4A7) to be very efficient in distinguishing between interior and coastal Douglas-fir. Thereby, nuclear microsatellites confirmed and verified the

results obtained with isoenzymes at the gene loci, 6-PGDH-A and PGM-A being the most discriminative (Li & Adams 1989, Leinemann 1998, Klumpp 1999). Seed stands in Bavaria exhibiting high proportions of allele A6 at loci 6-PGDH-A and PGM-A (>10% and >20%, respectively) are considered to contain the interior variety and thus are not considered for seed harvest. We were now able to transfer this system to microsatellite markers with high proportions of allele 96 at microsatellite locus PmOSU_3B2 and allele 272 at PmOSU_4A7, representing the interior gene pool. By using a Bayesian clustering approach based on both datasets, we were able to identify two subclusters, corresponding to coastal and interior Douglas fir. This helped us to identify the variety of the populations more successfully.

Thereby, we hope that this method will offer new possibilities to determine the origin of forest reproductive material of planted Douglas-fir stands in Europe more precisely. For two stands, we were able to identify the origin on a regional level (seed zone). For one stand this was not yet possible, to increase the number of reference stands from American seed zones might help in this case.

Based on isoenzyme markers, more than 70% of Douglas-fir stands in Bavaria have been characterized as pure coastal Douglas-fir, 2-3% as pure interior Douglas-fir and 25% of stands were mixed (Konnert et al. 2010). The pattern for intermediate populations can be interpreted as (i) mixed plantations consisting of the two varieties or (ii) a result of hybridization of coastal and interior type on an individual basis. However, currently we cannot clearly infer possible hybridization from our data, because we cannot distinguish the two varieties on the individual fully level. For such a purpose, a higher number of nuclear microsatellite loci distributed evenly across the whole genome and a higher discriminative power of each locus would be required (e.g. *Populus sp*., Lexer et al. 2005). We are aware, that four microsatellite loci is a rather low number of analyzed markers and should be increased in further studies. However, based on our results, we feel confident that the combination of isoenzymes and nuclear microsatellites is a very reliable method for the distinction of the two varieties. Quality issues (e.g. null alleles) and the ability to distinguish between varieties should be the main focus when developing and testing further microsatellite markers. Studies on species differentiation of oaks (e.g. *Q. robur* and *Q. petraea*: Neophytou et al. 2010, Gugerli et al. 2007, Gugerli et al. 2008) and poplars (Lexer et al. 2009) can serve as models for such approaches. New marker systems e.g. SNPs (single nucleotide polymorphisms) developed for Douglas fir (Howe et al. 2013) might also be an option for developing a set of markers for variety and provenance identification.

Uniparental inherited markers have recently been applied to reconstruct poleward Pleistocene migration of Douglas-fir populations in Mexico (Gugger et al. 2011), identifying large geographic structuring of populations. Mitochondrial markers, maternally inherited in Douglas-fir, showed distinction of the interior from the coastal variety (Gugger et al. 2011), but are not able to distinguish "grey" Douglas fir from the coastal type (unpublished results). Our reference populations from the transition zone (SA-BC, SL-BC) carried the same haplotypes as the reference populations representing the coastal type (e.g. CR-WA).

Among our studied populations, four were attributed to the interior Douglas-fir. However, SC-A-B and SC-NR-B on the one hand and FL-B, SA-BC and SL-BC on the other hand were clearly different in their genetic composition. Our explanation for such a pattern is that those populations originated from different regions within the extensive range of the interior Douglas-fir. Larger genetic differences between interior Douglas-fir populations compared to coastal ones, likely point out the more heterogeneous environment east of the Rocky Mountains, compared to the western part at

the Pacific coast. The fragmentation of forest land eastern of the Rocky Mountains might be responsible for the differences in phenological traits and growth rate (Kung & Wright 1972), and probably also for genetic differences.

While frequency differences at some isoenzyme and microsatellite loci might be considered as helpful measures for distinguishing the varieties, only a few diversity indices (N_E and H_O) were informative for that purpose in our study. Slightly higher genetic diversity was observed for interior Douglas-fir compared to the coastal type based on isoenzymes. This might be caused by more heterogeneous environment within the range of interior Douglas-fir as suggested by Krutovsky et al. (2009). The same authors further assumed that isoenzymes represent e.g. housekeeping genes and therefore the diversity and frequency differences among populations within coastal Douglas-fir are lower than those observed at nuclear microsatellite loci (Krutovsky et al. 2009).

The comparison of diversity measures for two populations (FR-A-B and SC-A-B) revealed that the transmission of genetic information from adults to natural regeneration seemed to be intact, with only slightly lower diversity values within the natural regeneration, compared to the adults in both stands. The genetic distances between adults and their natural regeneration were low in both studied populations. For isoenzymes, this finding was in accordance with the results of other studies on adult trees and natural regeneration of Douglas-fir (Konnert & Fussi 2012).

The intensive gene flow study in population FR-A-B showed that overall, a large proportion of adults participated in the reproduction, but some trees were more successful than others. Different flowering times of the adults and adaptation processes within the establishment phase of the young trees are likely the reasons for such observations. Phenological differences were also found to be responsible for differences in the mass pollination efficacy in a Douglas-fir seed orchard studied using a chloroplast DNA marker (Stoehr et al. 1998). Knowledge about reproductive patterns is of great importance for the management of Douglas-fir stands, since most of the established stands in Bavaria are not large continuous complexes, but were planted in small groups. Pollen exchange can be limited in such stands and might thus produce offspring with low genetic diversity. Moreover, differences in seed production of trees especially within the smaller plots might contribute to the finding, that the adult trees of the small plots contributed small amount of genetic information to the next generation. For example, the tree number 107 is a predominant tree, located at the border of plot AII, and might be especially fertile. Furthermore, some of the small groups might correspond to different provenances, which could also be responsible for the observed differences in reproductive patterns of different plots. Long distance pollen flow was reported to be very effective, as typical for wind-pollinated species (El-Kassaby & Davidson 1990). The amount of pollen flow was also studied by Slavov et al. (2005a) in seed orchards of *P. menziesii* in the USA, revealing a high pollen contamination of about 40%.

However, some cases exist in Bavaria, where groups of coastal and interior Douglas-fir were planted in close proximity. Natural regeneration in those stands might represent intervarietal hybrids or a mixture of both varieties, since it is known from artificial crossing experiments that the two varieties are interfertile (Rehfeldt 1977). Based on isoenzymes, Konnert et al. (2010) recorded that within one coastal Douglas-fir stand natural regeneration consisted mainly of interior Douglas-fir, due to gene flow from outside the stand. Although knowledge about the degree of participation of each variety to the reproduction within the stand is crucial due to its influences on the identity of the next generation, detailed gene flow studies within mixed stands do not yet exist. Such investigations should be planned in future studies, to assess possible hybridization

within artificially established mixed stands. In our study, no mixture of varieties was observed in the two analyzed stands including natural regeneration. However, almost 30% of pollen contribution from outside the stand has been recognized, despite a minimum distance to neighboring stands of 400 m.

Conclusions

The genetic differentiation between coastal and interior Douglas-fir can be improved using both isoenzyme and microsatellite markers, whereas small scale classification of provenances is still lacking appropriate genetic markers. In regions, where the coastal variety should predominate and start to naturally regenerate, removing interior Douglas-fir from neighboring stands is recommended. Currently, the results of our study have been implemented in the forest practice in Bavaria when seed harvesting stands are registered, during seed harvest and management planning of naturally regenerating stands.

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Appendix

Table S1 Diversity statistics for the four analyzed nuclear microsatellite loci. Actual allele number (N_A). effective allele number (N_E). allelic richness (A) after rarefaction (rarefaction size = 23), observed heterozygosity (H_O). expected heterozygosity (H_E) and fixation index (F_{IS}: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Pop	Locus	N	N_A	N_E	A	H_O	H_E	F_{IS}
CR-WA	3B2	87	32	19.1	22.5	0.839	0.951	0.145***
	4A7	88	42	21.1	25.6	0.830	0.956	0.159***
	3G9	83	12	4.7	10.7	0.735	0.832	0.350***
	2G12	87	25	12.3	17.0	0.837	0.925	0.143 n.s.
PF-WA	3B2	86	41	24.3	24.7	0.872	0.959	0.096***
	4A7	87	38	19.8	23.8	0.862	0.951	0.110***
	3G9	87	20	6.5	12.3	0.826	0.869	0.448***
	2G12	87	23	12.5	15.4	0.828	0.921	0.107**
SR-WA	3B2	88	37	16.4	23.7	0.818	0.946	0.195***
	4A7	88	37	22.8	24.2	0.795	0.959	0.221***
	3G9	87	14	6.8	12.4	0.805	0.876	0.238***
	2G12	88	18	7.4	13.0	0.864	0.867	0.009 n.s.
GR-WA	3B2	33	29	20.3	3.1	0.879	0.951	0.091*
	4A7	33	20	10.2	2.5	0.788	0.903	0.142**
	3G9	33	12	5.8	2.0	0.758	0.829	0.101 n.s.
	2G12	33	16	10.4	2.5	0.879	0.904	0.043 n.s.
ST-OR	3B2	41	33	23.6	3.3	0.927	0.958	0.045 n.s.
	4A7	36	28	17.7	3.0	0.694	0.944	0.277***
	3G9	37	11	5.9	2.0	0.757	0.832	0.104 n.s.
	2G12	39	24	11.1	2.7	0.795	0.910	0.140*
SA-BC	3B2	35	24	10.6	2.7	0.886	0.906	0.037 n.s.
	4A7	25	18	13.7	2.7	0.640	0.927	0.328***
	3G9	31	11	6.4	2.0	0.677	0.845	0.214**
	2G12	35	25	13.7	2.8	0.857	0.927	0.090*
SL-BC	3B2	27	20	9.7	2.6	0.815	0.897	0.110*
	4A7	24	18	12.9	2.7	0.583	0.923	0.386***
	3G9	25	12	7.4	2.1	0.720	0.866	0.189***
	2G12	27	20	13.2	2.7	0.815	0.925	0.137***
PH-B	3B2	96	36	21.3	3.2	0.906	0.953	0.054***
	4A7	83	31	19.0	3.1	0.795	0.948	0.167**
	3G9	79	14	7.1	2.2	0.772	0.859	0.108*
	2G12	88	22	10.8	2.6	0.818	0.908	0.104 n.s.
FL-B	3B2	39	32	19.0	24.6	0.846	0.947	0.120**
	4A7	34	21	10.4	18.6	0.735	0.920	0.330***
	3G9	39	19	6.1	16.6	0.684	0.873	0.340***
	2G12	37	25	17.1	20.9	0.811	0.942	0.152 n.s.
GB-B	3B2	51	27	15.9	20.4	0.824	0.937	0.131*
	4A7	51	26	10.8	18.6	0.824	0.914	0.146***
	3G9	52	10	5.4	9.8	0.769	0.853	0.420***
	2G12	51	15	8.4	11.6	0.824	0.882	0.076 n.s.

Table S1 (continuation)

Pop	Locus	N	N_A	N_E	A	H_O	H_E	F_{IS}
FA-B	3B2	38	27	16.9	21.8	0.868	0.941	0.091*
	4A7	37	25	14.4	20.9	0.757	0.935	0.230***
	3G9	37	17	9.8	14.6	0.946	0.899	-0.039 n.s.
	2G12	38	27	13.6	20.8	0.947	0.927	-0.009 n.s.
SC-A-B	3B2	53	25	6.2	17.9	0.811	0.841	0.045 n.s.
	4A7	53	23	11.5	17.2	0.811	0.917	0.142***
	3G9	52	12	6.9	10.9	0.788	0.872	0.201*
	2G12	51	20	11.6	16.9	0.804	0.922	0.194***
SC-NR-B	3B2	94	22	5.2	14.9	0.777	0.811	0.047 n.s.
	4A7	95	19	7.8	12.6	0.789	0.873	0.101**
	3G9	94	11	7.0	10.3	0.830	0.874	0.175**
	2G12	95	20	8.5	15.0	0.832	0.893	0.135**
FR-A-B	3B2	141	52	26.9	26.0	0.837	0.964	0.156***
	4A7	136	41	18.9	23.2	0.836	0.953	0.266***
	3G9	142	15	7.6	12.6	0.838	0.887	0.230***
	2G12	138	21	11.5	14.8	0.835	0.913	0.091***
FR-NR-B	3B2	242	48	26.6	25.8	0.868	0.962	0.100***
	4A7	241	40	14.4	20.5	0.884	0.938	0.191***
	3G9	242	15	6.0	10.4	0.810	0.862	0.363***
	2G12	240	21	11.1	13.6	0.838	0.910	0.082**